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Phenotypic and Functional Characteristics of FIV Infection in the Bone Marrow Stroma

Taishi Tanabe and Janet K. Yamamoto[1,2]

Department of Pathobiology, College of Veterinary Medicine, University of Florida, PO Box 110880, Gainesville, Florida 32611

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Human (HIV) and feline (FIV) immunodeficiency virus has been reported to infect bone marrow (BM) and stroma, followed by a loss in normal hematopoiesis. However, the magnitude and nature of HIV and FIV pathogenesis of the BM/stromal network are still unclear. In the current studies, pathogenesis of stromal cells was evaluated using the FIV model. Fourteen specific-pathogen-free cats inoculated with the four different strains (FIV_{UK8}, FIV_{Bang}, FIV_{Shi}, or FIV_{Pet}) were monitored for FIV infection in the peripheral blood mononuclear cells (PBMC), BM cells, and stromal cells. All inoculated cats became positive for FIV in the PBMC by 7 weeks p.i. and 13 of 14 cats had FIV in the BM cells by 7–13 weeks p.i. FIV was detected in macrophages and stromal fibroblasts from FIV_{UK8}-, FIV_{Bang}-, and FIV_{Shi}-infected cats but not from FIV_{Pet}-infected cats and only transiently in cells from FIV_{Shi}-infected cats. The ability of the supernatants from FIV-infected stromal cells to sustain the growth of uninfected BM cells was decreased 35–46% when compared to the supernatants from uninfected stromal cells. These results suggest that the FIV infection of the stroma alters normal hempatopoietic function(s) and that the infected stromal cells can also serve as a reservoir for FIV infection.

Key Words: feline immunodeficiency virus; FIV; AIDS; bone marrow stroma; stromal cells.

INTRODUCTION

Feline acquired immunodeficiency syndrome (feline AIDS) caused by feline immunodeficiency virus (FIV) is characterized by wasting, opportunistic infection, neutropenia, anemia, leukopenia, and a loss of immune function similar to that observed in human AIDS patients (Yamamoto *et al.,* 1988b). Peripheral blood cytopenias such as lymphopenia, monocytopenia, thrombocytopenia, and neutropenia are observed in most human immunodeficiency virus (HIV)-infected patients. The mechanisms leading to cytopenia of diverse hematopoietic lineages are still unclear. However, several potential mechanisms have been proposed including the loss of normal hematopoiesis caused by HIV infection of hematopoietic stem cells. Direct HIV infection of hematopoietic stem cells has been speculated to lead to the loss of the infected cells or to the disruption of their function(s) (Davis *et al.,* 1991; Folks *et al.,* 1988; Steinberg *et al.,* 1991). Alternatively, HIV may infect bone marrow (BM) stromal cells, which are vital in sustaining normal hematopoiesis. BM stromal cells consist of fibroblasts, endothelial cells, adipocytes, epithelial cells, and resident macrophages. These diverse populations of cells provide the microenvironment, such as growth factors, cellular interactions, and structural matrix, needed for proliferation and differentiation of hematopoietic stem cells. Several groups have reported that HIV is able to infect BM stromal cells and that these cells upon infection fail to provide growth factors to support normal hematopoiesis (Bahner *et al.,* 1997; Scadden *et al.,* 1990; Schwartz *et al.,* 1994). However, the mechanisms by which the virus affects the expression or release of the growth factors are not fully understood. Moreover, it is still unclear how many growth factors are affected by HIV infection of the marrow.

Although limited findings suggest a deleterious effect of HIV on the BM microenvironment, the lack of easy accessibility to BM and stromal samples from HIV-positive individuals has hampered the study of HIV pathogenesis in these tissues. As a result, the temporal events of pathogenesis in the BM microenvironment are still unclear. Animal models of AIDS have been frequently used in studies where samples from HIV-positive individuals are not readily available (Gardner and Luciw, 1989). Animal models also provide opportunity for an in-depth evaluation of temporal events that occur during lentiviral infection, including the determination of potential mechanisms by which these events ensue. The FIV model is an excellent small-animal model for studying lentiviral pathogenesis. FIV infection in cats also causes a loss of normal cellular composition of the marrow as well as BM dysfunctions that mimic those observed in HIV-positive patients (Pedersen and Barlough, 1991; Shelton *et al.,* 1990; Sparkes *et al.,* 1993). In a recent study, severe neutropenia observed during acute FIV infection was associated with the appearance of virus-

[1] J. K. Yamamoto is the inventor of record on a patent held by the University of Florida and may be entitled to royalties from companies that are developing commercial products that are related to the research described in this paper.

[2] To whom reprint requests should be addressed.

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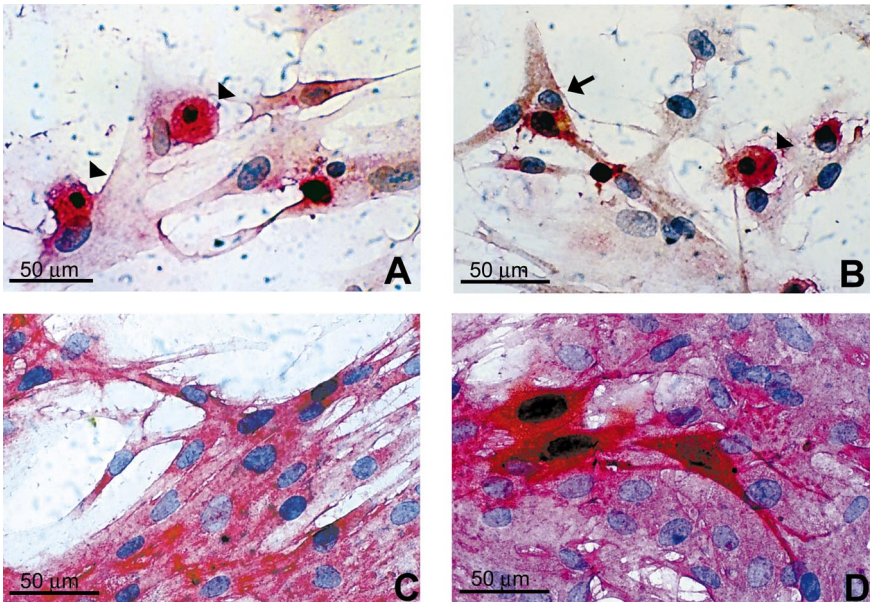

FIG. 1. Detection of FIV-infected stromal cells by dual-label staining. Stromal cells were stained with a combination of anti-FIV p24 antibody (brown stain) and either anti-macrophage or anti-fibronectin antibody (red stain). Round cells in the stromal cultures from uninfected cats to reacted with anti-macrophage antibody (red stain) but not with anti-FIV p24 antibody (▲, A), whereas cells from FIV-infected cats reacted with both anti-macrophage and anti-FIV p24 antibodies (dark reddish-brown staining, ▲, B). Spindle-shaped cells reacted with anti-FIV p24 antibodies (brown stain, ▲), but not with anti-macrophage antibodies (B). Spindle-shaped cells from uninfected stromal cultures reacted with anti-fibronectin antibody (red stain) but not with anti-FIV p24 antibody (C). In (D), all dark reddish-brown cells were staining positive for both anti-fibronectin (red stain) and anti-FIV p24 antibodies (brown stain).

infected BM accessory cells, such as macrophages (Beebe *et al.,* 1992; Linenberger *et al.,* 1995).

In the present studies, the FIV cat model of AIDS was used to study lentiviral pathogenesis in the BM microenvironment with specific emphasis on the susceptibility of BM stromal cells to FIV infection. The temporal infection of peripheral blood mononuclear cells (PBMC), BM cells, and stromal cells was evaluated using specific-pathogen-free (SPF) cats infected with four FIV strains. One goal of our studies was to determine whether the susceptibility of stem cells and stromal cells to FIV infection depends on the FIV strain infecting the host. Stromal cells derived from FIV-infected cats were evaluated for FIV infection, including the phenotype(s) and functional activity(s) of infected stromal cells. Functional activities of infected stromal cells were compared to those of uninfected stromal cells from age-matched SPF cats as a first step in identifying potential mechanisms by which FIV-infected cats develop impaired hematopoiesis.

RESULTS

Characterization of feline stromal cells

Primary feline stromal cells were isolated and established using the feline stromal cell culturing system, which is an adaptation of long-term bone marrow culture (LTMC) system described by others. (Dexter, 1979; Linenberger and Abkowitz, 1992). Stromal cells were characterized for their morphology and phenotypic characteristics after 21–28 days in culture, ≥7 days after gamma irradiation. Feline stromal cells consisted of two morphologically distinct populations of cells. The major population of cells was spindle-shaped cells, while a minor population of cells had round to more polymorphic morphologies. Based on immunohistochemical analysis, these primary stromal cells were uniformly negative for cytokeratin (an epithelial marker), von Willebrand's factor (an endothelial marker), CD45 (a leukocyte marker), and a pan-T cell marker (data not shown). Furthermore, the spindle-shaped cells reacted only with antibodies to vimentin (mesenchymal lineage marker) and fibronectin (fibroblast lineage marker) (data not shown). The minor population of round non-spindle-shaped cells reacted with antibodies to monocytes/machrophages (Figs. 1A and 1B) but not with antibodies to vimentin or fibronectin. Thus, the cultured feline stromal cells consisted of a major population of spindle-shaped fibroblasts and a minor population of macrophages. In addition, an immortalized stromal cell line (FeS-t) was derived from an uninfected SPF cat using the same stromal cell culturing system. FeS-t cells were uniformly spindle-shaped in morphology (Figs. 1C and 1D) and reacted with only antibodies to vimentin (data not shown) and fibronectin (Figs. 1C and 1D), suggesting that FeS-t cells are stromal cells of fibroblast lineage. Both primary stromal cells and FeS-t cells were used in the following studies to evaluate the susceptibility of the stromal cells to FIV infection.

TABLE 1

FIV Isolation from PBMC, BM Cells, and Stromal Cells of Cats Infected with 1000 CID_{50} of FIV_{UK8}

Cat	Subtype A strain	FIV isolation p.i. PBMC $[10^{-x}]$[a] 7 wk	PBMC 1.5 yr	Bone marrow cells $[10^{-x}]$ 7 wk	Bone marrow cells 1.5 yr	Stromal cells 7 wk	Stromal cells 1.5 yr
C1A	UK8	4	4	+[b]	4	+	+
C0B	UK8	4	5	+	4	+	+

[a] The number in the brackets represents the log of the highest dilution of infected cells in which FIV was detected after coculture with Con A-stimulated PBMC.

[b] + represents the FIV isolated from samples. However, these samples were not titrated.

FIV infection detected in the stromal cells from infected cats

Initially, FIV isolation was performed on primary stromal cells from experimental cats infected with 1000 cat median infectious dose (CID_{50}) of FIV_{UK8}. FIV was isolated from the PBMC, BM cells, and stromal cells at 7 weeks p.i., the earliest time point evaluated (Table 1). Both cats had an inversion of the CD4/CD8 ratio at 7 weeks p.i., and Cat C1A also developed a transient neutropenia at about the same time (data not shown). These cats displayed no other hematological or clinical symptoms throughout the study. The FIV isolation method we used detects the presence of FIV infection in the stromal cell population but can not identify the stromal cell type(s) that are infected with FIV. Consequently, primary stromal cells from infected Cats C1A and C0B were evaluated for the cell type(s) infected with FIV using dual-staining immunohistochemistry with anti-FIV p24 antibody and either anti-fibronectin or anti-macrophage antibody. FIV p24 was detected in extremely small numbers of either spindle-shaped cells reactive to anti-fibronectin antibody or round cells reactive to anti-macrophage antibody (Fig. 1). These results demonstrate that FIV was able to infect stromal fibroblasts and macrophages residing in the stromal microenvironment. Furthermore, FIV was still detectable in the stromal cells from these cats at 1.5 years p.i., suggesting that FIV infection of stromal cells is not transient.

In vivo and *in vitro* susceptibility of stromal cells to FIV infection

To determine whether the susceptibility of stromal cells to FIV infection *in vivo* depends on the viral strain or subtype, the stromal cells isolated from 12 cats experimentally infected with 10–100 CID_{50} of FIV_{UK8} (subtype A), FIV_{Pet} (subtype A), FIV_{Bang} (subtype B), or FIV_{Shi} (subtype D) were evaluated. The FIV inoculum consisted of pooled plasma or pooled PBMC isolated directly from previously infected cats. No cats displayed hematological or clinical symptoms throughout the study. All cats but one (Cat NJ2) had FIV infection in the PBMC and BM cells by 7 weeks p.i. (Table 2). At this early time point, FIV was detected in the stromal cells of only three cats (Cats 308, 295, and 315). However, FIV was detected in the stromal cells of 8 of 12 cats by 13 weeks p.i. Stromal cells from 2 of 2 cats infected with FIV_{Pet} and 1 of 2 cats infected with FIV_{Shi} never had detectable FIV infection even when FIV was isolated from the PBMC and BM cells (Table 2). To

TABLE 2

FIV Isolation from PBMC, BM Cells, and Stromal Cells of Cats Infected with Different FIV Strains

Cat	FIV Strain/subtype (CID_{50})	FIV isolation p.i. PBMC $[10^{-x}]$[a] 7 wk	PBMC 13 wk	PBMC 24 wk	PBMC 1 yr	Bone marrow cells $[10^{-x}]$ 7 wk	Bone marrow cells 13 wk	Bone marrow cells 24 wk	Bone marrow cells 1 yr	Stromal cells 7 wk	Stromal cells 13 wk	Stromal cells 24 wk	Stromal cells 1 yr
CU3	UK8/A (100)	3	3	2	3	+[b]	4	4	3	−	+	+	+
CW2	UK8/A (100)	3	2	2	2	+	3	3	2	−	+	+	+
308	UK8/A (100)	3	4	NA	NA	3	4	NA	NA	+	+	NA	NA
295	UK8/A (100)	2	3	NA	NA	2	4	NA	NA	+	+	NA	NA
DG4	Pet/A (100)	2	>2	−	>1	+	1	2	<1	−	−	−	−
NJ2	Pet/A (100)	2	1	1	1	−	2	1	2	−	−	−	−
CV5	Bang/B (100)	3	>4	3	4	+	4	3	2	−	+	+	+
CX5	Bang/B (100)	2	>2	NA	NA	+	1	NA	NA	−	+	NA	NA
315	Bang/B (10)	3	2	NA	NA	1	2	NA	NA	+	+	NA	NA
297	Bang/B (10)	<1	<1	NA	NA	3	<2	NA	NA	−	−	NA	NA
CT1	Shi/D (100)	2	>2	1	1	+	<1	1	1	−	+	+	−
NK2	Shi/D (100)	2	1	1	1	+	−	−	−	−	−	−	−

[a] The number in the brackets represents the log of the highest dilution of infected cells in which FIV was detected upon coculturing.

[b] + represents the FIV isolated from samples. However, these samples were not titrated.

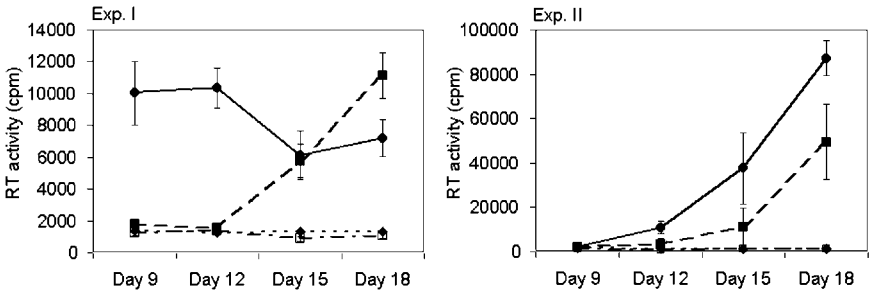

FIG. 2. FIV infection of FeS-t cells with or without direct contact with FeT-J/Bang cells. FeS-t cells were cocultured with mitomycin C-treated FeT-J/Bang cells (—●—) or cultured with FeT-J/Bang cells using transwell (- -■- -). After coculture, FIV isolation was carried out by RT assay as described under Materials and Methods. As additional controls, FIV isolation was also performed on Con A-stimulated feeder PBMC (- -◆- -) and untreated FeS-t cells (- -□- -).

determine the stability of the FIV infection in the stroma, a number of cats were monitored for 1 year. Three of the four cats (Cats CW2, CU3, CV5, and CT1) that were FIV positive in stromal cells at 13 weeks p.i. were still positive in stromal cells at 1 year p.i. However the one cat (Cat CT1) that became negative for FIV_{Shi} infection in the stromal cells at 1 year p.i. was still positive for FIV infection in the PBMC and bone marrow cells. These results demonstrate that FIV infection of PBMC and BM cells precedes infection of stromal cells. Unlike the transient FIV_{Shi} infection of stromal cells, the *in vivo* infection of stromal cells with FIV_{UK8} and FIV_{Bang} persisted throughout the year of study.

FIV strains from three different subtypes were used in this study. All subtypes were isolated from PBMC and BM cells of infected cats, except from BM cells of Cat NK2. FIV strains from all three subtypes were also isolated from stromal cells, although FIV_{Shi} from subtype D resulted in transient infection. Furthermore, FIV_{Pet}, which is subtype A the same as FIV_{UK8}, was not isolated from stromal cells. Therefore, the susceptibility of stromal cells *in vivo* to FIV infection depended on the FIV strain infecting the cat but was independent of the FIV subtype infecting the cat.

As another means to determine whether FIV susceptibility of stromal cells might depend on the viral strain infecting the cat, the susceptibility of feline stromal cells to infection *in vitro* with FIV_{UK8}, FIV_{Bang}, FIV_{Pet}, or FIV_{Shi} was evaluated using FeS-t cells. FeS-t cells were cocultured with mitomycin-C-treated FIV-infected cells (FeT-J/Bang, FeT-J/Shi, FL-4, or FIV_{UK8}-infected PBMC) for 3 days. After cocultivation, non-adherent cells were removed, and the adherent cells were cultured for one additional month before FIV isolation was attempted. All FIV strains were equally able to infect FeS-t cells *in vitro* (data not shown). Furthermore, to determine whether FIV infection requires direct contact with infected cells, FeS-t cells were cocultured directly with mitomycin-C-treated FeT-J/Bang cells or indirectly with untreated FeT-J/Bang cells using the transwell system, which separated the cells by porous membrane (0.4 μm diameter pores) (Fig. 2). FIV_{Bang} infected FeS-t cells with or without direct contact with infected cells. However, direct inoculation with cell-free FIV_{Bang} at even high doses (100–1000 median tissue culture infectious dose, $TCID_{50}$) did not result in the infection of FeS-t cells (data not shown). Hence, infection of FeS-t cells required the presence of infected cells but did not require the direct contact of the infected cells.

Loss of hematopoietic cell growth function of FIV-infected stromal cells

FIV-infected stromal cells were tested *in vitro* for their ability to support hematopoiesis. Culture supernatants from FIV-infected and uninfected stromal cells collected 4 days after a change of media ($\geq$1 week after irradiation) were tested for colony-forming ability using a methylcellulose culture system consisting of primary BM cells from SPF cats. In this assay, the number of colonies was measured as the level of growth promoting activity in supernatants. Stromal cells from four age-matched SPF cats were used to produce control supernatants (Table 3, control group). Colonies in the colony-forming unit (CFU) assay represent the proliferation or growth of granulocytes, monocytes, and megakaryocytes. The numbers of colonies from the BM cultures treated with stromal cell-supernatants from FIV-positive stromal cells were significantly lower than those from BM cultures treated with control supernatants ($P < 0.01$). Hence, the culture supernatants from FIV-infected stromal cells of Cats CU3, CW2, CV5, and CT1 at 24 weeks p.i. and Cats CU3, CW2, and CV5 at 1 year p.i. were unable to support the growth of hematopoietic cells at similar levels as the stromal-cell supernatants from SPF cats. The 35–46% decrease in colony numbers in the CFU assay correlated closely with the positive detection of FIV in the stromal cells. However, the supernatants from the FIV-negative stromal cells of FIV-infected cats (positive for FIV infection in the PBMC and BM cells) were able to support hematopoiesis at similar levels as the stromal-cell supernatants from SPF cats.

It has been reported that infectious HIV or HIV proteins

TABLE 3

Effect of FIV Infection on the Stem Cell

Source of stromal cells			FIV isolation from stromal cells		Number of colonies	
Cat	FIV strain	Subtype	24 wk p.i.	1 yr p.i.	24 wk p.i.	1 yr p.i.
Control[a]	–		–	–	53.8 ± 7.3[b]	28.5 ± 5.8
None[c]	–		–	–	4.0 ± 1.0	6.5 ± 0.5
CU3	UK8	A	+	+	34.7 ± 4.6[d]	18.0 ± 0.8[d]
CW2	UK8	A	+	+	29.0 ± 4.3[d]	17.3 ± 2.6[d]
CV5	Bang	B	+	+	32.7 ± 4.0[d]	18.3 ± 0.5[d]
DG4	Pet	A	–	–	39.3 ± 4.1	23.3 ± 5.8
NJ2	Pet	A	–	–	55.3 ± 9.0	27.7 ± 4.5
CT1	Shi	D	+	–	35.3 ± 3.9[d]	24.7 ± 4.7
NK2	Shi	D	–	–	51.0 ± 3.3	27.0 ± 5.0

[a] Control represents culture supernatants from stromal cells collected from four uninfected cats.
[b] The colony value represents the average of the mean colony numbers from four uninfected stromal-cell supernatants.
[c] None represents the cultures that received neither stromal-cell media nor 5% PHA–human-leukocyte-conditioned media.
[d] These values were significantly different from the control value at $P < 0.01$ using Student's two-tailed t-test.

may be the cause of decreased colony numbers in BM cultures (Calenda *et al.,* 1992; Folks *et al.,* 1988; Steinberg *et al.,* 1991; Zauli *et al.,* 1992a,b). Therefore, the stromal-cell supernatants used in the CFU assay were tested for FIV titer by direct RT assay and p24-ELISA. No stromal-cell supernatants had detectable FIV titer (data not shown). These results suggest that neither infectious FIV nor FIV proteins were the major factors inhibiting of hematopoietic cell growth.

DISCUSSION

Feline stromal cells established as LTMC consisted of a major population of fibroblasts and a minor population of macrophages. Our results agree with a previous report that the stromal cell types which predominate the feline LTMCs are fibroblastoid cells (50–80%) and macrophages (10–30%) at 3 weeks in culture (Linenberger and Abkowitz, 1992). Stromal cells obtained from FIV-infected cats (FIV_{UK8}, FIV_{Bang}, and FIV_{Shi}) were positive for FIV infection. Based on morphological and phenotypic characteristics, the infected stromal cells were identified as macrophages and stromal fibroblasts. FIV infection has been detected in macrophages residing in the BM microenvironment of both naturally and experimentally infected cats (Beebe *et al.,* 1992; Linenberger *et al.,* 1995). Therefore, it is possible that the stromal fibroblasts were infected with FIV from infected macrophages and infected T cells circulating in the marrow of the animal. However, it is also possible that the residual infected macrophages and infected T cells in the stromal preparation could have transmitted the FIV to the stromal fibroblasts during the establishment of LTMC. The possibility that infected T cells and other infected non-adherent cells transmitting the FIV to the stromal fibroblasts *in vitro* is low because non-adherent cells were removed by extensive washing as early as 1–2 days after seeding and irradiated after 1 week of culturing. In addition, hydrocortisone in the Iscove culture media and irradiation would have rapidly killed off the non-adherent lymphoid cells contaminating the stromal cultures. Since macrophages are less sensitive to irradiation and hydrocortisone, the possibility that infected macrophages transmitting FIV to stromal fibroblasts is higher than the transmission from infected non-adherent cells. Nonetheless, a large majority of infected stromal fibroblasts was not adjacent to or in close proximity to infected macrophages, suggesting that the infected macrophages in the culture were not the source of FIV for the infected fibroblasts. This view is supported by the findings of Linenberger *et al.* (1995), who detected FIV-infected macrophages in 2- to 3-week-old cultured adherent BM cells using *in situ* hybridization, whereas no FIV infection was detected in the stromal fibroblasts. If infected macrophages are the cell source of transmission, then infected stromal fibroblasts should have been detected in their study. Hence, we speculate that the *in vivo* transmission of FIV to stromal fibroblasts is more probable than *in vitro* transmission during the establishment of LTMC. This is the first report describing FIV infection of stromal fibroblasts in FIV-infected cats. Importantly, our findings agree with studies that demonstrate HIV infection of both BM macrophages and stromal fibroblasts (Gill *et al.,* 1996; Scadden *et al.,* 1990). HIV-1 infection has also been detected in stromal endothelial cells and myoid cells (Ercoli *et al.,* 1996; Moses *et al.,* 1996). In our stromal culture system, cell types other than macrophages and stromal fibroblasts (i.e., stromal endothelial, epithelial, reticular, and myoid cells) were not detected. Consequently, we could not determine the susceptibility of

feline stromal endothelial and myoid cells to FIV infection.

Studies were performed to evaluate the susceptibility of stromal cells to FIV infection *in vivo*. Age-matched SPF cats received *in vivo* inoculum of four different FIV strains, and their stromal cells were evaluated for FIV infection. Even under these controlled conditions, only two of four FIV strains (FIV_{UK8} and FIV_{Bang}) consistently infected the stromal cells *in vivo* and one other strain (FIV_{Shi}) caused transient infection. These results might show that FIV_{UK8} and FIV_{Bang} were able to infect stromal cells easily compared to FIV_{Shi} or FIV_{Pet}. Therefore, the susceptibility of stromal cells to FIV infection may depend on property of FIV strain but not subtype. Among the four FIV strains used in our studies, the FIV_{Shi} strain had the most T-cell tropism while FIV_{Bang} had strong cell tropisms for both T cells and macrophages (data not shown). FIV_{Pet} strain has been shown to have a strong T-cell tropism with moderate ability to infect monocyte/macrophages and microglial cells (Dow *et al.,* 1992). Although initial isolates of FIV_{Pet} strain were unable to infect both fibroblastic and macrophage-like cell lines (Pedersen *et al.,* 1987), long-term cultured FIV_{Pet} has been shown to productively infect Crandell feline kidney cells (CRFK), which consist predominantly of fibroblastic cells (Yamamoto *et al.,* 1988a). Based on these reports, our finding that the FIV_{Pet} strain was the most resistant to stromal cell infection *in vivo* was most unexpected. Perhaps the use of an *in vivo* derived inoculum in our studies may have contributed to this finding. It has been reported that *in vivo* derived FIV inocula are significantly different in cell tropism when compared to those of long-term cultured FIV (Bendinelli *et al.,* 1995; Hesselink *et al.,* 1999). Similar changes in tropism have been reported in primary HIV-1 isolates maintained in cultures for an extended time (Cheng-Mayer *et al.,* 1991; Wrin *et al.,* 1995). Thus, it is possible that the *in vivo* derived FIV_{Pet} is much more T-cell tropic and is similar in cell tropism to the original FIV_{Pet} isolates (Pedersen *et al.,* 1987). The T-cell tropic FIV_{Shi} strain also poorly infected stromal cells *in vivo*. Therefore, the inability of FIV_{Pet} to infect stromal cells *in vivo* may be linked to the preferential T-cell tropism of the stromal-resistant FIV strains. However, this conclusion needs to be explored more extensively.

In contrast to the *in vivo* findings, all four FIV strains were able to infect FeS-t cells *in vitro*. However, the *in vitro* infection of FeS-t cells was achieved with FIV-infected PBMC. The conflicting results between *in vivo* and *in vitro* FIV_{Pet} infection of stromal cells may be due to number of factors, such as: (1) the FeS-t cell line upon long-term culturing has developed diverse susceptibility to FIV strains, (2) the number of FeS-t like cells in the natural stromal cell population is low, or (3) *in vivo* pathogenesis in the BM microenvironment depends on the FIV strain infecting the host. The *in vivo* pathogenesis of BM/stromal network may involve factors that are not present in the *in vitro* stromal cell system. The dose of FIV to stromal cells, the infected cell types that interact with the stroma, and the immunity generated to the FIV may differ among FIV strains *in vivo* and cause a difference in the effect FIV infection has on the BM/stromal network. Since the FIV pathogenesis of lymphoid tissues and blood is closely associated with the strain infecting the animal (Dow *et al.,* 1999; Power *et al.,* 1998), it is also conceivable that strain difference also occurs during the pathogenesis of BM/stromal network. Furthermore, the *in vitro* infection of FeS-t cells did not require the direct contact with FeT-J/Bang cells since infection occurred even when separated by the transwell membrane. However, the presence of infected cells was required for FeS-t cells because cell-free FIV_{Bang} inoculum at even high doses was unable to infect the FeS-t cells. One possibility for this somewhat inconsistent observation may be that cytokines from the infected cells may augment the susceptibility of the FeS-t cells to virus infection. For example, interleukin-4 has been reported to induce cells to express CXCR4 (chemokine receptor that is a coreceptor for HIV) and render the cells more susceptible to HIV infection (Jourdan *et al.,* 1998; Valentin *et al.,* 1998). Another possibility is that the difference in the duration of viral exposure between the cell-free inoculum study (1-day exposure) and the infected-cell transwell study (3- to 6-day exposure) may have contributed to the inconsistent results. Hence, it may be possible that long-term exposure to cell-free virus eventually results in the infection of FeS-t cells.

Although more FIV strains need to be evaluated for stromal cell infectivity *in vivo*, our *in vivo* findings support the results from studies where macrophage-tropic HIV-1 strains were able to infect stromal cells more than those that were highly T-cell tropic (Schwartz *et al.,* 1994). However, a number of HIV studies conflict with this result. Macrophages in BM, which have been reported to be a major target for HIV infection, were susceptible to both lymphocytotropic and monocytotropic HIV strains *in vitro* (Gill *et al.,* 1996). A cell-free inoculum of lymphocytotropic $HIV_{IIIB,}$ but not of monocytotropic $HIV\text{-}1_{Ba\text{-}L}$ or $HIV\text{-}1_{RJ9533,}$ was able to infect stromal fibroblast cells *in vitro* (Scadden *et al.,* 1990). In contrast to the *in vivo* findings, our *in vitro* results suggest that stromal cell infection is independent of the cell tropism of FIV strain. Moreover, the susceptibility of the stromal cells to FIV infection both *in vitro* and *in vivo* was not associated in our studies with the FIV subtype infecting the cell or the host. Despite the conflicting results, one generalization that can be drawn from these studies is that the susceptibility of stromal cells to HIV or FIV depends heavily on the properties of the virus strain infecting the host.

Viral infection of the BM/stromal network might have a major influence on the level of hematopoiesis that occurs in the bone marrow of FIV-positive cats and HIV-positive

individuals. In our study, supernatants from FIV-positive stromal cultures from infected cats were unable to support hematopoiesis at levels equivalent to stromal-cell supernatants from SPF cats. A similar abnormality in hematopoietic function has been observed with HIV-infected stromal cells. A number of potential mechanisms for the abnormality in hematopoiesis have been proposed. One is that stromal-cell production of growth stimulator(s) or inhibitor(s) is affected by HIV infection of the stromal cells (Bahner *et al.,* 1997; Moses *et al.,* 1996; Schwartz *et al.,* 1995; Steinberg *et al.,* 1993). Another is that HIV protein or HIV infection of stem cells inhibits hematopoiesis (Calenda *et al.,* 1992; Folks *et al.,* 1988; Steinberg *et al.,* 1991; Zauli *et al.,* 1992a,b). In our study, none of the stromal-cell supernatants had viral RT activity or detectable FIV p24 protein. This suggests that neither infectious FIV nor FIV proteins are the major factors causing the loss of growth-promoting activity of the supernatants from infected stromal cells. It has been recently reported that FIV infection of feline stromal cell line (PNI cells), increased mRNA level of GM-CSF, IL-12 p40, IL-1β, stromal cell-derived factor-1 and hematopoietic inhibitors (TNF-α and MIP-1α), although the patterns of induction depended on the strain of FIV (Linenberger and Deng, 1999). Hence, other factors such as a loss or increase in production of growth stimulator(s) or production of growth inhibitor(s) by the infected stromal cells should be examined in future studies.

In summary, our results demonstrate that FIV can be detected in stromal cells from infected cats and that these infected stromal cells have the morphological and phenotypic characteristics of stromal fibroblasts. In addition, BM macrophages residing in close association with stromal cells are also infected with FIV. The susceptibility of the stromal cells to FIV infection depends on the strain of FIV infecting the animal. Moreover, FIV-infected stromal cells are unable to support the proliferation and/or differentiation of bone marrow stem cells. Our results also support the possibility that FIV-infected stromal cells are able to serve as a reservoir for viral infection. These observations closely mimic findings from HIV infection. Hence, the FIV model is a useful animal model for studying HIV pathogenesis of the BM/stromal microenvironment.

MATERIALS AND METHODS

Animals and FIV strains

Specific pathogen-free (SPF) cats were purchased from Liberty Research, Inc. (Waverly, NY) and Cedar River Laboratories (Mason City, IA). The animals were maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility with feed and water *ad libitum*. All SPF cats were confirmed to be negative for FIV by immunoblot analysis and by FIV isolation of their PBMC. SPF cats were inoculated intravenously (IV) with 10–1000 CID_{50} of FIV_{UK8} (Cats C1A, C0B, CW2, CU3, 308, and 295), FIV_{Pet} (Cats DG4 and NJ2), FIV_{Bang} (Cats CV5, CX5, 315, and 297), and FIV_{Shi} (Cats CT1 and NK2). All cats received 100 CID_{50} of FIV except for Cats 315 and 297, which received 10 CID_{50} of FIV_{Bang}, and Cats C1A and C0B, which received 1000 CID_{50} of FIV_{UK8}. The FIV_{UK8} inoculum administered to Cats C1A and C0B was pooled-infected tissue culture fluid from FIV_{UK8}-infected primary PBMC cultured for not more than 12 passages. The FIV inoculum for the remaining cats was in either pooled plasma or pooled PBMC that were collected directly from infected cats and were not cultured. FIV_{UK8} and FIV_{Pet} are strains belonging to FIV subtype or clade A. FIV_{Bang} and FIV_{Shi} belong to subtypes B and D, respectively. All cats were monitored for FIV titer in the blood and in bone-marrow aspirates at designated times post infection (p.i.). Four age-matched SPF cats were used as the source of uninfected stromal cells for producing control stromal-cell supernatants. Cats were anesthetized for BM aspiration or as needed for blood collection.

Establishment of stromal monolayers from long-term bone marrow cultures (LTMC)

Bone marrow cells isolated by centrifugation over Ficoll–Hypaque were used for establishing LTMC. The LTMC system used in our studies is modification of standard LTMC procedure (Dexter, 1979; Linenberger and Abkowitz, 1992). Isolated BM cells were washed three times in Hank's balanced salt solutions (HBSS) and resuspended in Iscove culture media consisting of Iscove's modified Dulbecco's medium (IMDM), 15% fetal calf serum (FCS), 15% horse serum, 10^{-4} mol/l 2-mercaptoethanol, 10^{-6} mol/l hydrocortisone, and 50 μg/ml Gentamicin. After 1–2 days in culture, the adherent cells were extensively washed with HBSS to remove nonadherent cells, were fed with fresh Iscove culture media, and were cultured until the cell monolayer was confluent. To remove residual hematopoietic cells, the trypsinized adherent cells were exposed to 1000 rad of gamma irradiation and then seeded into flasks. Cells were cultured for additional 1–2 weeks before their use in assays or analysis. Using the preceding procedure, a feline stromal fibroblast cell line (FeS-t cells) was established from an SPF cat.

Characterization of feline stromal cells

Stromal cells grown on chamber slides (Lab Tek, Nunc, Naperville, IL) were used for immunohistochemistry. Stromal cells on the chamber slides were fixed with 50% acetone and 50% methanol for 2 min. The slides were washed with phosphate-buffered saline (PBS) and immunohistochemical analysis was performed with ABC universal kit by Vector Laboratories, Inc. (Burlingame, CA) using the manufacturer's procedure. The dual-label

protocol consisted of enzymatic reaction with Vector Red substrate (red staining) followed by 3,3′-diaminobenzidine substrate (brown staining). Rabbit anti-rat fibronectin (Chemicon International, Temecular, CA), mouse anti-human vimentin (Serotec Inc., Raleigh, NC), mouse anti-human desmin (Serotec Inc.), mouse anti-feline CD45 (Serotec Inc.), mouse anti-feline pan-T antigen (kindly provided by Dr. Nazareth Gengozian, Department of Pediatrics, University of Tennessee), mouse anti-human monocyte/macrophage antigen (Serotec Inc.), rabbit anti-bovine cytokeratin (Dako Corp., Carpinteria, CA), rabbit anti-human Von Willebrand Factor (Dako Corp.), and mouse anti-FIV p24 (East-Coast Biologics, Inc., North Berwick, ME) antibodies were used as either phenotype-specific antibodies or FIV-specific antibodies.

FIV isolation from PBMC, BM, and stromal cells

FIV isolation from PBMC and BM cells was performed as previously described (Yamamoto *et al.,* 1998). BM cells or PBMC isolated by centrifugation over Ficoll–Hypaque were serially diluted in RPMI culture media and then seeded into 24-well plates or 25-cm^2 flasks. RPMI culture media consisted of RPMI 1640, 10% FCS, 100 U/ml human IL-2, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 50 μg/ml Gentamicin. PBMC from uninfected SPF cats were stimulated with 2 μg/ml concanavalin A (Con A) in RPMI culture media for 3 days and then were recultured every 3 days for 1–2 weeks in fresh RPMI culture media without Con A. These Con A-stimulated PBMC were used as highly FIV-susceptible feeder cells. The feeder cells (1×10^6 cells/well of 24-well plate or 5×10^6 cells/25 cm^2 flask) were cocultured with serially diluted BM cells or PBMC from FIV-infected cats. Supernatants from cocultures were collected every 3 days and replaced with fresh RPMI culture media. These supernatants were assayed for RT activity using a previously described RT assay (Rey *et al.,* 1984). Irradiated stromal cells were cultured for at least one additional week. Then cells were trypsinized and seeded in 25-cm^2 flasks at 1×10^6 cells/flask. After overnight incubation, a total of 5×10^6 feeder cells was added to the stromal cell culture, and cocultured in RPMI media supplemented with 20% FCS. Culture supernatants were collected every 3 days and assayed for RT activity.

In vitro infection of stromal cells

FeS-t cells were seeded at 1×10^6 cells/25 cm^2 flask and cultured overnight to monolayer confluency of 60–75% confluence. In one set of studies, the monolayer cells were cocultured for 3 days with 5×10^6 cells/25 cm^2 flask of mitomycin C-treated FIV-infected cell lines or mitomycin C-treated FIV_{UK8}-infected PBMC. FIV-infected IL-2-independent T-cell lines used in our experiments were FeT-J/Bang, FeT-J/Shi, and FL-4 (Yamamoto *et al.,* 1991, 1998). FL-4 is a T-cell line chronically infected with FIV_{Pet}. After coculture, the monolayer cells were extensively washed and cultured for one additional month before coculturing with Con A-stimulated uninfected PBMC for FIV isolation as described above. In another set of studies, the FeS-t cells were cultured in the presence of 100 or 1000 $TCID_{50}$ of cell-free FIV inoculum for 24 h. The FIV inocula used were $FIV_{Bang,}$ $FIV_{UK8,}$ FIV_{Pet}, and FIV_{Shi}, their titers were based on a titration performed on primary PBMC. After incubation with cell-free virus, the monolayer cells were washed, resuspended in fresh culture media, and cultured for ≥1 week before FIV isolation using the method described above.

To determine whether FIV infection require direct contact with infected cells, FeS-t cells were seeded at 2.5×10^5 cells/well of 12-well plate and cultured overnight. Transwell system (Corning Costar Corp., Cambridge, MA) allows cells in membrane-separated chambers to be cultured in the same fluid environment without direct contact. FeT-J/Bang cells were added into the upper chamber of the transwell and cultured for 3–6 days with FeS-t cells placed in the lower chamber. As a positive control, mitomycin C-treated FeT-J/Bang cells were cocultured directly with FeS-t cells. After culturing, FeS-t cells were washed, trypsinized, seeded into 25 cm^2 flasks, and cultured for ≥4 weeks. Cells were then trypsinized, reseeded into 24-well plates at 1×10^5 cells/well, and cultured until monolayer confluency of 60–75%. Subsequently, Con A-stimulated uninfected PBMC (feeder cells) were added to the FeS-t cells and FIV isolation was performed as described above.

Colony-forming unit assay (CFU assay)

The ability of stromal cells to support hematopoiesis was determined by CFU assay. This assay was modified and performed as previously described (Linenberger *et al.,* 1991; Schwartz *et al.,* 1995). Briefly, uninfected BM cells were cultured at 10^5 cells/dish in Iscove's methylcellulose media supplemented with 10% cell-free stromal-cell supernatant derived from stromal monolayer cultures from either FIV-infected or uninfected cats. The Iscove's methylcellulose media consisted of IMDM, 0.9% methylcellulose, 30% FBS, 10^{-5} M 2-mercaptoethanol, and 5% PHA-human leukocyte conditioned media (StemCell Technologies Inc., Vancouver, Canada). All stromal-cell supernatants were collected from stromal monolayer cultures 4 days after the previous culture media change and were free of viable cells by filtration or by freeze-thawing. CFU assays were set up in triplicate and colonies ≥40 cells in a cluster were counted after 10 days of culturing. The colonies obtained from CFU assay were composed predominantly of granulocyte/monocyte colonies and few of these colonies also contained megakaryocytes. Erythroid colony was not observed in our studies because erythropoietin was not supplemented to the cultures.

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